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Research article

Cytological observation of anther development of cytoplasmic male sterility and thermosensitive genic male sterility systems in rice

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Abstract

Male sterility is an excellent tool for use in hybrid rice production as it involves naturally occurring emasculation of the male gamete, thus preventing self-pollination in plant species. There are two male sterility systems—cytoplasmic male sterility (CMS) and thermosensitive genic male sterility (TGMS) —being widely used in rice breeding programs. Although TGMS and CMS systems are valuable tools for rice breeding, and the genetic and molecular mechanisms in various plants have been extensively studied, knowledge of the critical stage of cellular change in the rice anther is still imprecise. To gain an understanding of the actual crucial developmental stage of the rice anther that affects pollen productivity, cytological events were observed throughout the anther developmental process in both TGMS (KUT1) and CMS (IR80151A) rice lines. The results revealed that male sterility in the KUT1 line was most pronounced in the late meiotic stage during the developmental process and ultimately resulted in empty locules that were without pollen grains. In the IR80151A line, male sterility was displayed at the late vacuolated stage and consequently caused pollen abortion. Therefore, it was concluded that male sterility of CMS and TGMS occurs post meiosis.

Introduction

Hybrid rice is an excellent solution for increasing rice yield due to its vigor, higher yield potential beyond that of its parents. However, it is well known that the limitation of hybrid rice production is the self-pollination characteristic of rice (FAO, 2004). For several decades, rice breeders have vigorously researched to discover easier techniques for emasculation of the male gamete to produce hybrid rice (Chen et al., 2007). Male sterility is a spontaneous emasculation in plant species, which is a desirable tool for using in rice breeding because it produces non-functional pollens or abnormal pollens that are incompatible with the female gamete (Hanson and Bentolila, 2003). Therefore, plant breeders have extensively applied the male sterility system to F_1 hybrid production in various species (Sheng et al., 2015). Two male sterility systems that are frequently used in plant breeding are cytoplasmic male sterility (CMS) and thermosensitive genic male-sterile (TGMS) (Sheng et al., 2015). CMS is a maternally inherited trait through a mutation in the mitochondrial genome that

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leads to the production of dysfunctional pollens (Ku et al., 2003; Wang et al., 2006). It has been observed in over 150 plant species and is frequently associated with unusual open reading frames (ORFs) leading to premature cellular degradation of the tapetum which results in the suppression of viable pollen production (Ku et al., 2003; Eckardt, 2006; Luo et al., 2006; Wang et al., 2006; Fu et al., 2008). A type of CMS line that has been repeatedly used to produce F_1 hybrid is the wild-abortive CMS (WA-CMS) line (Huang et al., 2014). The WA-CMS line produces pollen abortion which often occurs in early microspore development, in particular at the uninucleate stage (Luo et al., 2013). Unlike CMS, TGMS is a male sterility system where sterility of the male gamete is controlled by thermo-variation. For example, in rice, temperatures exceeding 30°C can produce male-sterile gametes, while temperatures below 26°C induce male-fertile gametes (Ku et al., 2001; Kalaiyarasi and Vaidyanathan, 2003). Male sterility of TGMS occurring pre or post meiotic division or both results in different male-sterile manners (Ku et al., 2001, 2003; Kalaiyarasi and Vaidyanathan, 2003). In addition, the tapetum has an important role in anther development because it is involved in anther programmed cell death development and microspore formation, including producing essential proteins for pollen formation. Some reports have revealed that the cause of TGMS male sterility in rice involves programmed cell death which arises in the premeiotic stage and results in vacuolated-tapetal cells development before normal programmed cell death of the tapetum so that ultimately, anther locules become empty locules (Ku et al., 2001, 2003; Kalaiyarasi and Vaidyanathan, 2003). Tapetum degeneration is typically triggered by the programmed cell death process during the late pollen mitosis stage in which the *TDR* gene is involved in both tapetum development and degeneration (Li et al., 2006). In addition to the *TDR* gene, *Ugp1* (UDP-glucose pyrophosphorylase 1) is also essential for pollen mother cell meiosis and microspore development in the rice anther, and the suppression of *Ugp1* causes pollen degeneration (Chen et al., 2007). In Thailand, hybrid rice has been extensively studied, particularly the TGMS and CMS systems (Tan et al., 1998). Although there is genetic and molecular evidence that reveals the cause of male sterility in both systems, cytological confirmation is limited. Therefore, the current study divulged the sequential cytological events which occurred during anther development in TGMS and CMS through rice germplasm in order to address the cause of male sterility occurrence in rice.

Materials and Methods

Plant materials

Rice seeds of two male sterile lines—TGMS (KUT1) and CMS (IR80151A-wild-abortive cytoplasmic male sterility system; WA-CMS)—and two male fertile lines—IR80151B (a maintainer line) and CH4 (a restorer line) —were sown in a seed tray. The rice seedlings were then transplanted into an 20 cm × 30 cm pots and maintained in a greenhouse for three weeks under natural conditions (higher than 30°C), except for the TGMS line that was grown in a separate growth chamber set (daily mean temperature 20°C). The panicles of each line were used in this experiment to observe the actual developmental stage of the anthers. At the flowering stage, three tillers from each variety were sampled to examine the anther and type of pollen abortion. Anthers were observed under a stereomicroscope and pollen fertility was determined by using 1% I_2-KI solution staining. Plants with no stained pollen were classified as completely male sterile, whereas plants having more than 95% darkly stained pollen were classified as male fertile (Sreewongchai et al., 2014).

Specimen fixation, infiltration and embedding

The panicles from each experimental line were harvested for microscopic analysis. To speculate the developmental stage of the anther, the spikelets were appraised by the different length of anthers. They were then dissected and the anthers were pre-fixed in 2.5% glutaraldehyde solution at 4°C overnight and then rinsed three times with 0.1 M sodium-potassium phosphate buffer (Na-K phosphate buffer, pH 7) for 10 min, and post-fixed in 1% osmium tetroxide solution for 1 hr at 4°C, before rinsing three times with distilled water. The specimens were dehydrated using a graded EtOH series, then replaced with *n*-butyl glycidyl ether for 1 hr. Spurr's resin was infiltrated into the specimens for 1 hr, and then repeated. All specimens were finally embedded in resin blocks which were fully filled with Spurr's resin and polymerized in an oven at 65°C overnight.

Microscopic analysis

To obtain semi-thin sections for microscopic analysis, the specimens were sectioned into 200–250 μm thickness using an ultra-microtome (model Leica Ultracut UCT-GA-D/E-1/100; Nakhon Pathom, Thailand), and were stained with 3% Toluidine Blue O in distilled water. Specimens were observed under a light microscope (model CX31; Olympus; Nakhon Pathom, Thailand). Fresh anthers from each experimental line were observed under a stereo microscope.

Results

Phenotypic observation of male-fertile and male-sterile lines

To distinguish the male-sterile phenotype between CMS and TGMS, the phenotypes IR80151A as well as the KUT1 lines were observed and compared with the IR80151B and CH4 lines throughout the growth stages. The results showed no differences in their phenotypes at the early reproductive stage (the panicle initiation stage toward the booting stage) in all experimental lines. The late reproductive stage in the IR80151A and KUT1 lines exhibited the male-sterile phenotype with pale, shrunken anthers within the spikelets at the flowering stage (Figs. 1I and 1J, respectively) and had no seeds at the ripening stage (Figs. 1E and 1G, respectively). In contrast, the IR80151B and CH4 lines contained yellow, plump anthers within the spikelets (Fig. 1H) and had a higher seed-setting.

To confirm pollen fertility, the pollen grains from each line were stained with I_2-KI solution and the results are displayed in Fig. 1K. The anthers of the IR80151B and CH4 lines contained numerous darkly stained, rounded pollen grains, whereas those of the IR80151A line displayed many light brown, shrunken pollens, lacking starch accumulation capability while the KUT1 line had no pollen within its anthers (Figs. 1L and 1M, respectively). Although the IR80151A line produced mature pollen grains, they were dysfunctional pollens. These results indicated that KUT1 and IR80151A differed in their male-sterile characteristics. Furthermore, at the ripening stage, no significant difference was observed in plant phenotype such as plant height and leaf shape among the IR80151A and IR80151B (Fig. 1A) because the IR80151B line is an isogenic line of IR80151A. The phenotype of the CH4 and KUT1 lines differed slightly as the CH4 showed erect leaves whereas the KUT1 has straight leaves (Figs. 1B and 1C, respectively).

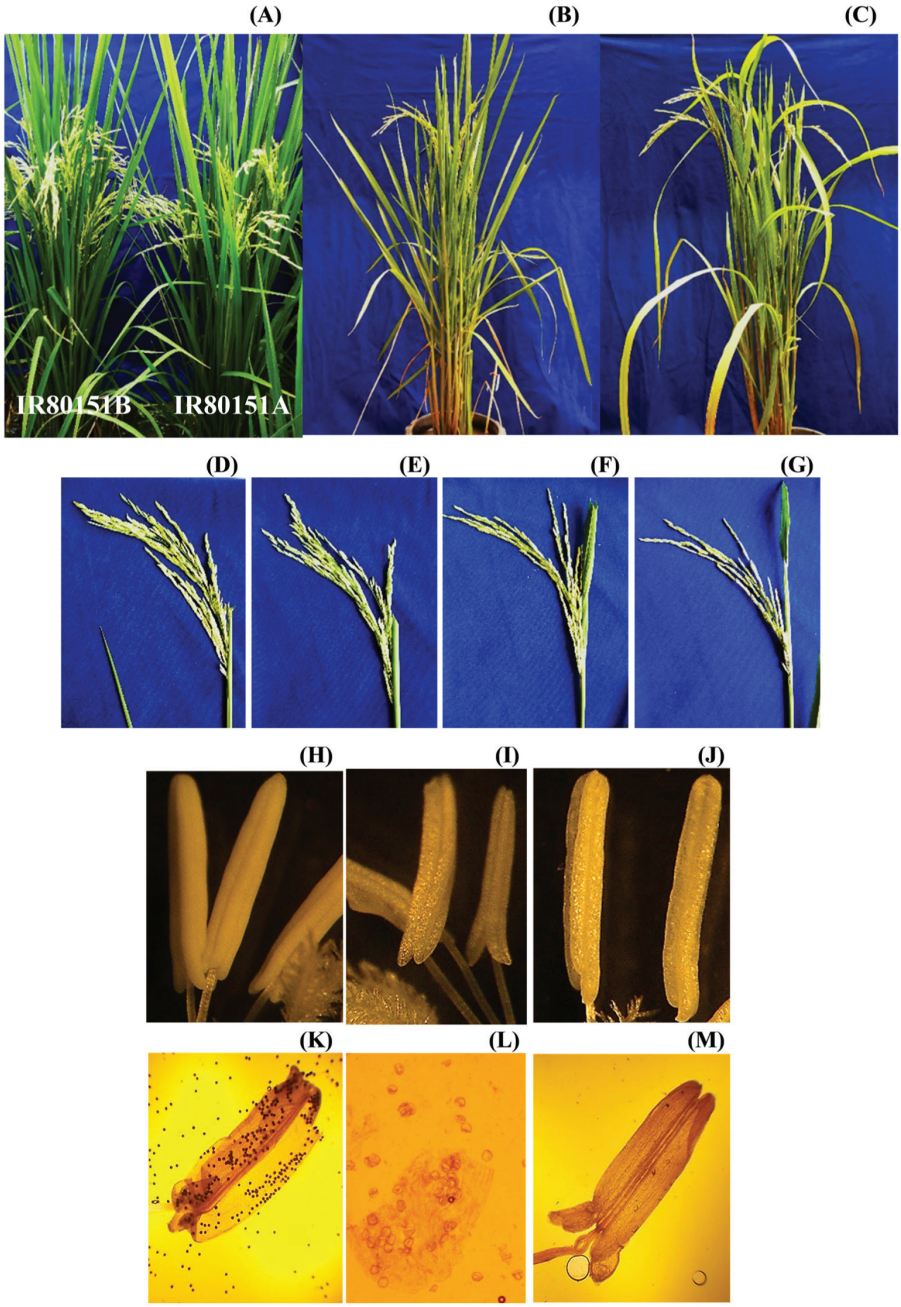

Fig. 1 Phenotypes of four experimental lines (IR80151B, IR80151A, CH4, KUT1): (A)–(C) Mature plants: (A) IR80151B (left) and IR80151A (right); (B) CH4; (C) KUT1. (D) –(G) Mature panicles: (D) IR80151B; (E) IR80151A; (F) CH4; (G) KUT1. Anther morphology of fertile and sterile lines: (H) IR80151B; (I) IR80151A; (J) KUT1. (K) –(M) Anther and pollens stained with I_2-KI solution: (K) IR80151B; (L) IR80151A; (M) KUT1

Cellular changes in male gamete of the two male-sterile lines

To examine the actual cellular change of the anther during its developmental process in the two male-sterile lines, the cytological events were observed that occurred during the developmental process of the anther in the two male sterile lines (IR80151A and KUT1) compared with the two male fertile lines (IR80151B and CH4) as the control. The spikelets from each experimental line were dissected and the anthers collected for microscopic observation. The anthers were preserved using fixatives and embedded into Spurr's resin for transverse section, cut into slices with 200–250 µm thickness using an ultra-microtome and stained with Toluidine Blue O before observation under a light microscope. Itoh et al. (2005) divided the cellular change of anther development into eight stages; however, the current study divided its development into seven stages as shown in Fig. 2. In this experiment, the examination of cellular change in rice anthers was observed throughout its developmental process using semi-thin sections. The results showed that there was no difference in the anther locules in the male-sterile lines and the male-fertile lines from prior to the meiosis stage. At the microsporogenesis stage the four layers of the anther wall (epidermis, endothecium, middle layer, tapetum) were typically formed within the locules and underwent several mitotic divisions of the primary parietal cells, while the primary sporogenous cells (PSCs) went through several mitotic divisions (Figs. 2A–D). The PSCs were then differentiated into pollen mother cells (PMCs) after the completion of the anther four-wall layers (Figs. 2E–H). The PMCs underwent meiotic division and ultimately released free tetrads within the anther locules. At the tetrad stage, the tapetal cells became darkly stained, and nucleuses within the tapetal cells of the two male-fertile lines were clearly visible, while the tapetal cells of the two male-sterile lines only showed dark staining (Figs. 2I and 2L). Furthermore, the endothecium cells were vacuolated and the middle layers were degraded in the anther locules of all lines (Figs. 2I–L). At the late meiosis stage, an abnormality appeared in the KUT1 locules, the microspores within the KUT1 anther locules ruptured and released cytosol into the locules (Fig. 2O). Conversely, the locules of other lines were normal with spherical microspores (Figs. 2M–N and 2P). This abnormality continued toward the vacuolated stage, during which the ruptured-microspores were consistently degraded and within the KUT1 anther locules, the rectangular tapetal cells and endothecium layer were also initially degraded and later became an empty-rectangular tapetum in many cells, while the endothecium layer still remained (Fig. 2S), whereas in the other lines it became an amorphous, tapetal layer and the endothecium layer totally disappeared (Figs. 2Q–R and 2T). Consequently, the anther locules of KUT1 were completely empty at the pollen mitosis stage (Fig. 2W) Furthermore, the vacuolated-endothecium layer was visible in its locule in the pollen mitosis and mature pollen stages (Figs. 2W–ZI). In contrast to KUT1, the microspores of IR80151A, IR80151B and CH4 underwent mitotic division and formed immature pollens. The immature pollens of IR80151B and CH4 shapes were globular and distorted, respectively, and were filled with reserve substances (Figs. 2U and 2X), unlike those of IR80151A which were vacuolated and deformed (Fig. 2V). Furthermore, the tapetal layer of IR80151A disappeared while that of IR80151B and CH4 was almost totally corrupted. Later, the immature pollens of CH4 reformed from the distorted shape to a spherical shape like IR80151B, containing cytosol and ultimately developed into mature pollens (Figs. 2Y and ZII), whereas those of IR80151A became unfilled mature pollens. Eventually, the IR80151A locules released dysfunctional pollens (Fig. 2Z). The cytological results indicated that the male sterility of the IR80151A line was due to pollen abortion at the late vacuolated stage, whereas that of the KUT1 line occurred at the uninucleate stage during the developmental process.

Discussion

Phenotypic characteristics of CMS and TGMS lines in rice

Based on the phenotypic observation of rice male-fertile lines (IR80151B and CH4) and male-sterile lines (IR80151A and KUT1) in this research, there were no obvious differences before the heading stage. Later, the male-sterile characteristics were observed at the flowering stage during which the anther locules of IR80151A contained dysfunctional pollen grains while those of KUT1 presented empty locules (Figs. 1L and 1M). The results indicated that the male sterility processes of male gametes in CMS and TGMS were different. The TGMS lines derived from diverse genetic backgrounds had different types of microspore formation (Kalaiyarasi and Vaidyanathan, 2003). Hence, the genetic background may affect the development of the male gamete in different ways. The sterility of male gametes in most TGMS lines was largely induced by higher temperature (more than 25°C), which is the critical temperature for prompting the male sterility (Ku et al., 2001, 2003; Kalaiyarasi and Vaidyanathan, 2003; Lopez et al., 2004; Shankar et al., 2007). In addition to high temperature, chilling also affects rice anther development resulting in male sterility at the booting stage (Mamun et al., 2006).

Cytological analysis during anther development of CMS and TGMS lines in rice

Cellular events occurring during anther development in rice have been classified into seven or eight stages (Itoh et al., 2005; Li et al., 2006; Chen et al., 2007). The current study, based on the cellular incidents observed under the optical microscope, seven stages of anther development were identified. From prior to the meiosis stages during the anther developmental process, there were no differences in any of the experimental lines. Typically, anther development in rice initiates as archesporial cells that undergo periclinal division to construct the primary parietal layer and establish the microsporangial walls which then form the primary sporogenous layer. The sporogenous layer develops to meiocytes, and the meiocytes undergo meiosis to form tetrads of haploid microspores. Cytoplasmic streaming in anther locules primarily occurred when they had developed young microspores and vacuolated-pollen. Later, the tapetal cell layer was simultaneously differentiated and degraded. At the mature pollen

stage, trinucleate pollens developed through mitotic division, became mature pollen grains and then were released from the locules by rupturing of the endothecial cell layer (Jung et al., 2005). Meiosis is the major event in which meiocytes are transformed to microspores during anther development (Mamun et al., 2005). TGMS is a male sterility system which is controlled by thermo-variation. The critical event of male sterility in TGMS was initially observed in the late meiosis stage in KUT1 locules, in which the tapetal cell layer was dense and thick. Some young microspores showed distorted shapes and initially ruptured. Later, the young microspores of KUT1 were extensively broken and released cytosol into the locules space (Fig. 2O). Although the tapetum-dense cell layer remained in the locules, the occurrence of ruptured microspores was continuous within the KUT1 locules and ultimately resulted in empty locules (Fig. 2S). The results indicated that the male sterility of KUT1 (TGMS line) resulted from the failure of microspore formation. The sterility-control genes usually function in any of the three anther development stages of pre-meiosis, meiosis and post-meiosis Therefore, the abnormal function of those genes affects pollen formation during the anther developmental process and leads to various phenotypic manners of male sterility in plants (Kalaiyarasi and Vaidyanathan, 2003) such as suppression of the *Ugp1* gene, a homolog gene of the *UGPase* gene in rice, resulting in the collapse of the pollen formation initially observed in the early meiosis stage (Chen et al., 2007). Therefore, the male sterility of anther development in the TGMS line probably relates to either preprogramed cell death of the tapetal cells or carbohydrate metabolism-regulated genes. In contrast to the TGMS line, in CMS the male sterility system is a maternally inherited trait, in which an unusual open reading-frame occurs in the mitochondrial genomes (Eckardt, 2006). The current study elucidated the critical cellular change in the IR80151A anther. The IR80151A line is a wild abortive type CMS (WA-CMS) which produces dysfunctional pollen grains. The crucial stage which led to pollen abortion during anther development in the IR80151A line occurred in the pollen-mitosis stage with vacuolated-pollens. Ultimately, at the mature pollen stage, they became empty mature pollens without any nutrients. Although the CMS anthers contained numerous empty pollen grains, the stomium of the CMS locules still degraded to release pollen grains resembling the locules of male-fertile lines. At the vacuolated-pollen stage toward the pollen-mitosis stage in the IR80151A line, the tapetal cell layer degraded normally as shown in the male-fertile lines. Consequently, the crucial event for male sterility in both the TGMS and CMS lines occurred in different stages of anther development.

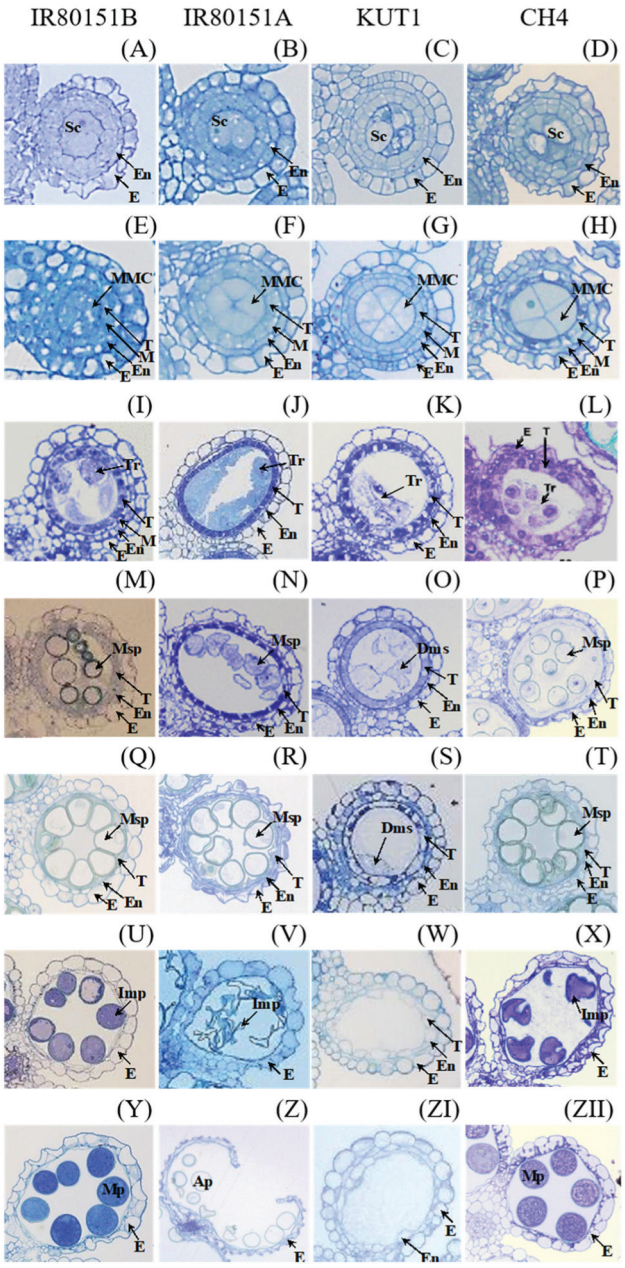

Fig. 2 Major events of cellular change during anther development. Microscopic observation of anther development of four experimental lines (IR80151B, IR80151A, KUT1, CH4): (A)–(ZII) showing one of the four locules in transverse section of all lines in different developmental stages of anthers: (A)–(D) pre-meiosis stage; (E)–(H) early meiosis stage; (I)–(L) late meiosis stage; (M)–(P) young microspore stage; (Q)–(T) vacuolated-pollen stage; (U)–(X) pollen-mitosis stage; (Y)–(ZII) mature pollen stage, where E = epidermis; En = endothecium; Dms = degraded microspore; Imp = immature pollen; M = middle layer; MMC = microspore mother cell; Mp = mature pollen; Msp = microspore; Sc = sporogenous cell; T = tapetum; Tr = tetrads

Conflict of Interest

The authors declare that there are no conflicts of interest.

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